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Hydrolysis of orange peel by a pectin lyase-overproducing hybrid obtained by protoplast fusion between mutant pectinolytic *Aspergillus flavipes* and *Aspergillus niveus* CH-Y-1043

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ABSTRACT

Pectin lyases cleave the internal glycosidic bonds of pectin by β-elimination, producing non-saturated galacturonic oligomers. Genetic improvement of pectin lyase-overproducing strains is still necessary to improve industrial processes based on this enzyme. In the present study hybrids were obtained by protoplast fusion between mutant pectinolytic *Aspergillus flavipes* and *Aspergillus niveus* CH-Y-1043 strains. Prototrophic segregants showed different isoenzymatic profiles and produced increased levels of pectin lyase in cultures containing lemon peel as a sole carbon source. Hybrid HZ showed an increase of 450% and 1300% in pectin lyase production compared with that of *A. niveus* CH-Y-1043 and *A. flavipes*, respectively. Pectin lyase produced by the hybrid HZ was partially purified and used for the hydrolysis of orange peel. Pectin lyase was able to hydrolyze 56% of orange peel biomass. However, addition of 2 RFU and 20 U of endo- and exo-polygalacturonase, respectively, induced the hydrolysis of 92% of orange peel solids. In conclusion HZ is a pectin lyase-overproducing hybrid with potential applications in the pectin industry.
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1. Introduction

Pectin is a complex heteropolysaccharide present in higher plant tissues. Pectin is made of a linear chain of poly-α-(1-4)-D-galacturonic acid with varying degree of methylation of the carboxylic acid residues. This primary backbone, known as the "smooth region", is interrupted by heavily branched regions of rhamnogalacturonan (hairy region). The biological breakdown of pectin is catalyzed by the pectinolytic enzymes. These enzymes are widely used in the industrial processing of fruits [1].

Pectinolytic enzymes are classified into two major groups: methylesterases, which remove methoxyl groups, and depolymerases (hydrolases and lyases), which cleave the bonds between galacturonate units. Pectin lyases (EC 4.2.2.10) are enzymes with the capacity to depolymerize the smooth region of pectin by cleaving internal glycosidic bonds by β-elimination, generating non-saturated pectin oligomers containing a terminal non-reducing residue with a 4,5-double bond. Pectin lyases have been identified in fungi of the genus *Aspergillus* [2–4]. Screening, and genetic improvement of new pectin lyase-overproducing strains is still necessary to improve industrial processes based on this enzyme. Gene transfer between fungi species by protoplast fusion has been shown to be an effective procedure for the improvement of *Aspergillus* strains with increased production of extracellular enzymes [5,6].

In a previous work, we reported the generation of interspecific hybrids showing a modest increment in the production of extracellular pectin lyase. These prototrophic hybrids were obtained by protoplast fusion between auxotrophic mutants of pectinolytic *Aspergillus* strains [7]. However, it has been demonstrated that induction of haploidization of heterozygous diploids obtained by protoplast fusion, may produce segregants with greater variations in their morphology and enzyme productivities [8]. Thus, in the present study haploidization by *p*-fluorophenylalanine was induced in prototrophic fusion products obtained by protoplast fusion between pectinolytic strains of *Aspergillus*. Haploid segregants were then tested for their capacity to produce extracellular pectin lyase using various agroindustrial by-products as carbon source. A pectin lyase-overproducing hybrid was detected, and its potential use for the hydrolysis of orange peel was assessed.

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2. Materials and methods

2.1. Materials

Malt Extract Agar was obtained from Difco (Detroit, USA). Citric pectin, *p*-fluorophenylalanine (PFP), diphenylamine reagent, NADP, α-naphthyl acetate, Fast Garnet GBC Base solution, β-naphthyl phosphate sodium salt, Fast Blue RR salt, and 3,5-dinitrosalicylic acid (DNS) reagent were obtained from Sigma Chemical Company (MO, USA). Czapek-Dox Agar, Potato Dextrose Agar (PDA), glucose, yeast extract, peptone, and agar were obtained from Merck (Darmstadt, Germany). All the reagents used for analysis of proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories (CA, USA). Lemon, orange, grapefruit and passion fruit peels were obtained from local fruit processing industries.

2.2. Microorganisms

Aspergillus flavipes was obtained from ATCC (ATCC 16795). *Aspergillus* sp. CH-Y-1043 was isolated from soil as previously described [9]. *A. flavipes* and *Aspergillus* sp. CH-Y-1043 were maintained and propagated by monthly transfers to PDA plates at 29 and 37 °C, respectively, and stored at 4 °C.

2.3. Identification of microorganisms

Identification of parental strains was achieved by cultural, morphological and molecular methods. The isolates were grown on Czapek-Dox Agar and Malt Extract Agar to confirm their purity and species identity based on macro- and micro-morphological criteria [10]. Identification was subsequently confirmed by sequencing the Internal Transcribed Spacers 1 and 2 (ITS1-ITS2) of the nuclear rRNA by using the ascomycete-specific primer pair ITS5 (5′ GGA AGT AAA AGT CGT AAC AAG G 3′) AND ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′). DNA was extracted as previously described [11]. Amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, CA, USA) as described elsewhere [12] with minor differences in the thermal cycling conditions employed: initial denaturation at 94 °C for 1 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 2 min, and 72 °C for 1 min. A final extension step at 72 °C for 5 min was then conducted. All PCR products were purified and sequenced on both strands using the primers described above on an automated capillary DNA sequencer ABI Prism 310 genetic analyzer (Applied Biosystems, CA, USA). After sequencing the identity of *A. flavipes* was confirmed, whereas the strain CH-Y-1043 was identified as *Aspergillus niveus*.

2.4. Protoplast fusion and haploidization

Auxotrophic mutants *Aspergillus* A13 (*ade*$^-$) and *A. flavipes* F78 (*lys*$^-$), derived from *Aspergillus niveus* CH-Y-1043 and *A. flavipes*, respectively, were used as fusion partners in the protoplast fusion experiment as previously described [13]. Auxotrophic mutants were grown in complete medium (2% (w/v) glucose, 0.3% (w/v) yeast extract, 0.3% (w/v) peptone, 2% (w/v) agar). Fusion products were selected on minimal medium (2.0% (w/v) glucose, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4{\cdot}7H_2O$, 0.05% (w/v) KCl, 0.001% (w/v) $FeSO_4$, 0.3% (w/v) $NaNO_3$, 2.0% (w/v) agar, pH 4.5). To induce haploidization, regenerating heterokaryotic colonies were transferred to complete medium containing 1 mg mL^{-1} PFP and incubated at 29 °C for 15 days. Putative haploid recombinants were purified by serial sub-cultivation in complete medium containing PFP. Colonies that demonstrated to be stable after several cycles of sub-culturing on PFP-containing plates were isolated and characterized. Stability of selected segregants, as well as their production of extracellular enzymes was repeatedly tested during the course of this study.

2.5. Determination of DNA content

DNA content was determined in conidia from parental and hybrid strains. DNA was extracted from 1×10^9 conidia as reported previously [11], and estimated by using the diphenylamine reagent method [14]. Calf thymus DNA was included as standard.

2.6. Isoenzyme analysis

Total intracellular protein was extracted from parental and hybrid strains growing in complete medium. Mycelia were resuspended in 3 mL buffer (0.1 M Trizma-base, 33 mg NADP, 1.2 mM EDTA). Cells were frozen using liquid nitrogen, thawed at 37 °C, and ground in a mortar. Cell debris was removed by centrifugation at 10,000 rpm for 20 min at 4 °C. Protein extracts were lyophilized and stored at −20 °C until used. Total protein content of the lysate was determined by the Bradford method [15]. 20 μg of protein were resolved on a vertical 7.5% SDS-PAGE for 4 h at 100 V. Alkaline and acid phosphatases in the gel were stained according to the method described by Shaw and Prasad [16]. Briefly, after electrophoresis, the gels were incubated in a staining solution for acid phosphatase [90 mL Tris–HCl buffer (pH 5.8), 1 mL of 0.2 M $MgCl_2$, 100 mg α-naphthyl acetate, 1 mL Fast Garnet GBC Base solution], or for alkaline phosphatase staining [90 mL of 0.1 M Tris–HCl buffer (pH 8.1), 100 mg β-naphthyl phosphate sodium salt, 100 mg Fast Blue RR salt, 1 mL of 0.2 M $MgCl_2$, 150 mg $MgSO_4$] for 2 h at 37 °C. The gels were then distained in a 1:2:1 (v/v) solution of methanol:distilled water:acetic acid.

2.7. Production of extracellular enzymes

Lemon peel, passion fruit peel, and grapefruit peel were used for the induction of pectinases because of their high content of pectin (35.0%, 18.4%, and 21.2%, respectively) [17–19], and also because they are produced in large quantities as waste from the fruit processing industry. The peels were ground using a food processor, and then washed three times with double-distilled water. Peels were dried at 50 °C for 24 h before being utilized as substrate for the production of extracellular enzymes. Parental and hybrid fungi were grown in 500 mL Erlenmeyer flasks containing 200 mL of liquid medium [0.2% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) KH_2PO_4, 0.2% (w/v) K_2HPO_4, pH 3] supplemented with either citric pectin, lemon peel, passion fruit peel, or grapefruit peel at a concentration of 1% (w/v). The medium was autoclaved at 121 °C for 20 min. The flasks containing medium were inoculated with 2×10^6 spores mL^{-1}. Cultivation was carried out at 37 °C, on a rotary shaker (New Brunswick Scientific Co., NJ, USA) at 200 rpm, for 120 h. Samples were collected at 24-h intervals during 120 h. Samples were centrifuged for 20 min at 2500 rpm at room temperature to remove spores. Supernatants were filtered through Millipore 0.45 μm membranes, and kept frozen at −20 °C until used. These preparations were used as enzyme-containing filtrates during the course of the study.

2.8. Determination of extracellular enzymatic activities

Pectin lyase (EC 4.2.2.10) activity was assayed spectrophotometrically by measuring the formation of 4,5-unsaturated oligogalacturonates at 235 nm [20]. The reaction mixture containing 1 mL of 0.05 M Tris–HCl buffer (pH 8.8), 1 mL of 0.8% (w/v) citric pectin and 0.5 mL of the enzyme-containing filtrates or purified fractions was incubated at 45 °C for 60 min. The reaction was terminated by addition of 4.5 mL of 0.01N HCl. Absorbance at 235 nm was determined in a spectrophotometer GeneQuant pro (Amersham Biosciences, Buckinghamshire, UK). Enzyme activity was expressed as Units (U). One pectin lyase unit was defined as the amount of enzyme that increased the absorbance by 0.1 in 1 h at 235 nm.

Endo-polygalacturonase (EC 3.2.1.15) activity was determined by the decrease of viscosity of a citrus pectin solution. The reaction mixture contained 0.5 mL of either enzyme-containing filtrate or purified fraction, and 10 mL of 1% (w/v) citrus pectin solution in sodium acetate buffer (pH 4.2), and was incubated at 30 °C for 60 min. Viscosity-diminishing activity was determined by measuring the decrease in relative viscosity of the citrus pectin solution using an Ostwald viscometer (Kimax. IL, USA) with a water flow of 10 s at 30 °C. Enzyme activity was expressed as relative fluidity units (RFU). One RFU is equivalent to the amount of enzyme required to decrease by 1.0 the relative fluidity per second.

Exo-polygalacturonase (EC 3.2.1.67) activity was evaluated by measuring the liberation of reducing sugars. Soluble reducing sugars released were estimated by the DNS method using galacturonic acid as standard [21]. The reaction mixture containing 0.5 mL of either enzyme-containing filtrate or purified fraction and 1 mL of 1.0% (w/v) citrus pectin in sodium acetate buffer (pH 5.5), was incubated at 45 °C for 60 min. The reaction was terminated by adding 3 mL DNS reagent. Absorbance at 550 nm was determined in a spectrophotometer GeneQuant pro (Amersham Biosciences, Buckinghamshire, UK). Enzyme activity was expressed as Units (U). One enzyme unit is defined as the amount of enzyme that releases 1 μM of reducing sugars per minute under assay conditions.

2.9. Effect of pH and temperature on pectin lyase activity

The effect of pH on pectin lyase activity was determined using 0.5 mL of purified enzyme, diluted in 1 mL of McIlvaine buffer at pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0, and supplemented with 1% (w/v) lemon peel. The mixture was incubated at 50 °C for 60 min. To measure the effect of temperature on pectin lyase activity, the reaction mixture was prepared using McIlvaine buffer at the pH in which the maximum activity of pectin lyase was demonstrated. Incubation was performed at 10, 20, 30, 40, 50, 60, and 70 °C for 60 min. Pectin lyase activity was determined as described above.

2.10. Purification of pectin lyase

A 5-day-old submerged culture of pectin lyase-producing strains was centrifuged for 20 min at 6500 rpm at 4 °C, and subsequently clarified by filtration. The culture filtrate was used as an enzyme source for purification experiments. The crude enzyme was concentrated to one-tenth of its original volume by tangential flow ultrafiltration with a 10 kDa membrane under constant pressure using a Pellicon Laboratory System (Millipore Corporation, MA, USA). Concentrated proteins were precipitated with cold acetone. Precipitated proteins were resuspended in 0.02 M ammonium acetate buffer (pH 5.0). The concentrated enzyme was loaded onto a DEAE-Sephadex A-50 column previously equilibrated with 0.02 M ammonium acetate buffer (pH 5.0). After washing with the same buffer, adsorbed pectin lyase was eluted at a flow rate of 0.5 mL min^{-1} with a linear gradient of 0–0.5 M NaCl in 0.02 M ammonium acetate buffer (pH 5.0). Protein concentration and pectin lyase activity were measured in all collected fractions. Eluted fractions were analyzed by

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